Clinical implications of the cross-talk between renin-angiotensin-aldosterone system and vitamin D-FGF23-klotho axis

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ASSOCIATIONS OF 25(OH) AND $1,25(OH)_2$ VITAMIN D WITH LONG-TERM OUTCOMES IN STABLE RENAL TRANSPLANT RECIPIENTS

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ABSTRACT

Context. Vitamin D deficiency is common in renal transplant recipients (RTR). The long-term implications of vitamin D deficiency in RTR remain unclear.

Objective. We investigated whether 25(OH) or 1,25$(OH)_2$ vitamin D levels are associated with mortality, renal function decline, and graft failure in stable RTR.

Design. Observational study with longitudinal design. Follow-up was 7.0, interquartile range (IQR) 6.2-7.5 years.

Setting. Single-center outpatient clinic.

Participants. 435 stable RTR (51% men, mean age 52±12 years) were included at a median [IQR] of 6 [3-12] years after kidney transplantation.

Main Outcome Measures. All-cause mortality, annual change of estimated glomerular filtration rate (eGFR), and graft failure.

Results. Mean 25-hydroxyvitamin D [25(OH)D] and 1.25-dihydroxyvitamin D [1,25$(OH)_2$D] were 21.6±9.1 ng/mL and 45.2±19.0 pg/mL, respectively. During follow-up, 99 patients (22.8%) died and 44 patients (10.1%) developed graft failure. In univariable analysis, both 25(OH)D and 1,25$(OH)_2$D were significantly associated with mortality (hazard ratio [HR], 0.64; 95% confidence interval [CI], 0.51-0.81; *P*<.001 and 0.69 [95% CI, 0.55-0.87], *P*=.002 per SD increase, respectively). The inverse association of 25(OH)D with mortality remained significant after adjustment for potential confounders (HR 0.68 [95% CI, 0.52-0.89], *P*=.004 per SD increase). The associations of 1,25$(OH)_2$D with mortality and graft failure lost significance after adjustment for renal function. Severe vitamin D deficiency (25[OH]D <12 ng/mL) was independently associated with stronger annual eGFR decline .

Conclusions. Low 25(OH)D is independently associated with an increased risk of all-cause mortality and 25(OH)D <12 ng/mL with a rapid eGFR decline in stable RTR. The association of low 1,25$(OH)_2$D with mortality or graft failure depends on renal function. These results should encourage randomized controlled trials evaluating the effect of vitamin D supplementation after kidney transplantation.

INTRODUCTION

Kidney transplantation improves the prognosis and quality of life of patients with end-stage renal disease and is therefore considered the preferred treatment for most patients with end-stage renal disease. Although short-term prognosis after kidney transplantation has strongly improved over the past decades, on the long run, even stable renal transplant recipients remain at strongly increased risk of premature death compared with the general population.[1] Therefore it is crucial to identify modifiable factors affecting long-term outcomes after kidney transplantation.

Vitamin D is a hormone that regulates serum calcium and phosphate levels by stimulating intestinal absorption of calcium and phosphate and by stimulating renal calcium reabsorption. Vitamin D deficiency contributes to secondary hyperparathyroidism and subsequently to mineral and bone disorders, but has also been implicated in extraskeletal adverse conditions including hypertension, insulin resistance, renal function loss, congestive heart failure, and mortality.[2-6] Vitamin D is hydroxylated to 25-hydroxyvitamin D [25(OH)D] in the liver and afterwards converted into its active form, 1,25-dihydroxyvitamin D [1,25$(OH)_2$D], by the enzyme 1α-hydroxylase. The enzyme 1α-hydroxylase is predominately but not exclusively found in renal tubular epithelial cells. In kidney disease, the renal capacity to generate 1,25$(OH)_2$D is progressively lost due to reduced 1α-hydroxylase activity in the kidney.[7,8]

Several studies have shown that 25(OH)D deficiency is common after kidney transplantation, partly due to avoidance of direct sunlight exposure to reduce the enhanced risk of nonmelanoma skin cancer caused by immunosuppressive treatment.[9-12] A recent study documented that low levels of 25(OH)D, but not 1,25$(OH)_2$D, measured 3 months after transplantation are associated with an increased risk of renal interstitial fibrosis, renal tubular atrophy, and lower glomerular filtration rate (GFR) 1 year after kidney transplantation.[13] However, vitamin D levels may vary considerably along with renal function, fibroblast growth factor 23 (FGF-23), and PTH during the first year after transplantation. Consequently, vitamin D status early after transplantation may not represent vitamin D status during later stages post transplantation. The aim of the current study was to investigate whether vitamin D status, measured in a cohort of stable renal transplant recipients (RTR) more than 1 year after transplantation, is associated with long-term patient and graft outcomes.

MATERIALS AND METHODS

Research design and subjects

In this prospective observational single-center cohort study, all adult stable RTRs who visited our outpatient clinic between August 2001 and July 2003 and had a functioning graft for greater than 1 year were invited. Patients with overt congestive heart failure or cancer other than cured skin cancer were considered ineligible for the study. A total of 606 of 847 eligible RTRs (72%) signed written informed consent. For this post-hoc analysis, vitamin D status [*i.e.*, 25(OH)D and

1,25(OH)$_2$D] was measured in 437 RTRs (72.1%). In this study we included 435 subjects (99.5%) with 25(OH)D levels greater than 4 ng/mL. The main characteristics (*i.e.*, age, sex, renal function, systolic BP, waist circumference, number of events) of subjects that were not included in this post-hoc analysis were not materially different from the 435 RTRs who were included in this study. Additional details of this study have been published previously.[14-18] The institutional review board approved the study protocol (METc 2001/039), which was in adherence to the Declaration of Helsinki.

Study population

The Groningen Renal Transplant Database contains information on all renal transplantations performed at our center since 1968. Relevant transplant characteristics, such as donor age, donor sex, human leukocyte antigen mismatches, and date of transplantation were extracted from this database. Current medication was taken from the medical record. Alcohol consumption, smoking status, and cardiovascular history were obtained using a self-report questionnaire. Cardiovascular history was defined as a history of myocardial infarction, percutaneous transluminal angioplasty or stenting of coronary or peripheral arteries, bypass operation of coronary or peripheral arteries, claudicatio intermittens, amputation for vascular reasons, transient ischemic attack, or an ischemic cerebrovascular accident. Body mass index, waist circumference, and BP were measured as described previously.[15] Diabetes mellitus was diagnosed if the fasting plasma glucose concentration was at least 7.0 mmol/L (≥126 mg/dL) or antidiabetic medication was used.

Clinical endpoints

The primary endpoint of this study was all-cause mortality. Secondary endpoints were annual change in eGFR and death-censored graft failure defined as return to dialysis or retransplantation. The continuous surveillance system of the outpatient program ensures up-to-date information on patient status and cause of death. General practitioners or referring nephrologists were contacted in case the status of a patient was unknown. Endpoints were recorded until the end of May 2009; median follow-up was 7.0; interquartile range (IQR), 6.2-7.5 years. Cause of death was obtained by linking the number of the death certificate to the primary cause of death as coded by a physician from the Central Bureau of Statistics. There was no loss due to loss of follow-up.

Laboratory measurements

Upon entry in the cohort, blood was drawn after an 8-12 hour overnight fasting period. EDTA plasma and serum samples were stored at -80°C until assessment of biochemical measures for this study. Vitamin D status was assessed by measuring 25(OH)D in EDTA plasma using isotope dilution–online solid phase extraction liquid chromatography tandem mass spectrometry[19], 1,25(OH)$_2$D was measured by liquid chromatography tandem mass spectrometry[20]. Plasma C-terminal FGF-23 levels were determined by sandwich ELISA (Immutopics).[21] Plasma creatinine concentrations were determined using a modified version of the Jaffe method (MEGA AU 510,

Merck Diagnostics) until March 2006, and thereafter by an enzymatic assay (Roche). A correction factor (calculated using Passing and Bablok linear regression [CLSI EP9 guideline]) was applied to adjust values that were measured by the Jaffe method. eGFR was calculated using the chronic kidney disease Epidemiology Collaboration (CKD-EPI) equation.[22] Annual change in the eGFR was calculated from the slope of the regression line through all available eGFR values during follow-up (provided that a minimum of three values were available). PTH was measured using a RIA. Serum albumin, calcium, cholesterol, C-reactive protein (CRP), glucose, hemoglobin, phosphate and triglycerides, and urinary total protein were determined by routine laboratory measurements.

Statistical analysis

Data are presented as mean ± SD, median [interquartile range], and number (percentage) for normally, non-normally distributed data, and nominal data, respectively. $P<0.05$ (two-tailed) was considered to indicate statistical significance. Statistical analyses were performed using SPSS 20.0 for Windows (SPSS Inc.), R Foundation for Statistical Computing, and Stata Statistical Software: Release 11.

All baseline data were greater than 99% complete except for PTH (21% missing values). Multiple imputation (n=5) was used to deal with missing baseline data.[23] Variable distribution was tested with histograms and probability plots. For illustrative purposes, the study population was subdivided into tertiles of baseline vitamin D status to visualize associations with vitamin D. *P* for differences in vitamin D tertiles were assessed with ANOVA for normally distributed continuous data, the Kruskal-Wallis test for non-normally distributed data, and the χ^2 test for nominal data. Univariable and subsequent multivariable linear regression analyses were used to identify independent determinants of 25(OH)D or $1,25(OH)_2D$ levels. Non-normally distributed variables were transformed to the natural log to fulfill criteria for linear regression analyses. Multivariable linear regression models were constructed using backward selection ($P_{out}>0.05$) including variables that were significantly associated with plasma 25(OH)D or $1,25(OH)_2D$ levels in univariable analysis.

Associations of 25(OH)D and $1,25(OH)_2D$ with all-cause mortality were assessed using Cox proportional hazards regression analysis with adjustment for age, sex, current smoking, systolic BP, waist circumference, diabetes mellitus, eGFR (CKD-EPI) and determinants of 25(OH)D (*i.e.*, season, low-density lipoprotein [LDL] cholesterol, ln PTH, and dialysis vintage) and determinants of $1,25(OH)_2D$ (*i.e.*, high-density lipoprotein [HDL] cholesterol, use of angiotensin-converting enzyme inhibitor/angiotensin receptor blocker [ACEi/ARB], phosphate, and transplant vintage), respectively.

The multivariable-adjusted Cox regression analyses for the associations of 25(OH)D and $1,25(OH)_2D$ with death-censored graft failure were adjusted for (recipient) age, (recipient) sex, human leukocyte antigen mismatches, donor age, donor sex, type of kidney transplantation (living or deceased), eGFR (CKD-EPI), (ln transformed) proteinuria, and determinants of 25(OH)D (*i.e.*, sex, season, LDL cholesterol, PTH, and dialysis vintage) and determinants of $1,25(OH)_2D$ (*i.e.*,

current smoking, HDL cholesterol, use of ACEi/ARB, serum phosphate, and transplant vintage), respectively. Continuous variables were entered as continuous variables in the models. Cox regression models were built stepwise to avoid overfitting and to keep the number of covariates accurate in relationship to the number of events.[24]

The association of vitamin D levels with annual change in eGFR was assessed using linear regression analyses. Given that the association of 25(OH)D with annual change in eGFR was not linear, we categorized 25(OH)D levels in two categories (*i.e.*, <12 ng/mL versus ≥12 ng/mL [30 nmol/L]) based on the results of the fractional polynomial regression analysis. In multivariable linear regression analyses, we adjusted for potential confounders including age, sex, waist circumference, systolic BP, diabetes, baseline eGFR, and (ln transformed) proteinuria.

Because vitamin D supplementation could influence the association between vitamin D status and outcomes, we excluded subjects using vitamin D supplements at baseline in additional sensitivity analyses. We repeated the Cox regression analyses for all-cause mortality and death-censored graft failure and the linear regression analyses for annual change in eGFR.

RESULTS

Study population

The study population consisted of 435 RTRs (51% male, aged 52 ± 12 y) at a median time of 6.3 (interquartile range [IQR], 3.1-11.7) years after kidney transplantation. Mean concentrations of 25(OH)D and $1,25(OH)_2D$ were 21.6 ± 9.1 ng/mL (54.0 ± 22.8 nmol/L) and 45.2 ± 19.0 pg/mL (108.4 ± 45.6 pmol/L), respectively. In this population, 214 patients (49%) were vitamin D deficient [25(OH)D <20 ng/mL], 145 (33%) patients were insufficient (20-30 ng/mL) and 76 (18%) were sufficient (>30 ng/mL). Baseline patient characteristics of the study population are shown in Table 1. Baseline characteristics according to tertiles of 25(OH)D and $1,25(OH)_2D$ are presented in Supplemental Table 1. At baseline, 43 patients (10%) were using vitamin D supplements; 34 patients used alfacalcidol with daily dose 0.25 [IQR, 0.25-0.5] µg, three patients used calcitriol with daily dose 0.25 or 0.5 µg, two patients used dihydrotachysterol with daily dose 0.1 or 0.2 mg, and for four patients the type of vitamin D analog was unknown. Multivariable linear regression analyses showed that $1,25(OH)_2D$, season, sex and LDL cholesterol were positively associated, whereas waist circumference, PTH and dialysis duration were inversely and independently associated with 25(OH)D concentrations (Table 2). In similar analyses, eGFR, 25(OH)D, use of statins and HDL cholesterol were positively associated, whereas transplant vintage, serum phosphate, use of ACEi/ARB and smoking were inversely and independently associated with $1,25(OH)_2D$ levels (Table 2).

Vitamin D status and mortality

At a median follow-up period of 7.0 (IQR, 6.2-7.5) years, 99 of 435 patients died (22.8%). In univariable analyses, both 25(OH)D and $1,25(OH)_2D$ were significantly associated with all-cause

Table 1. Baseline patient characteristics of the study population and associations with 25(OH)D and 1,25(OH)$_2$D levels

	Patient characteristics	Univariable associations with vitamin D (*P* for trend)	
Characteristic	**(n=435)**	**25(OH)D**	**1,25(OH)$_2$D**
25(OH)D, ng/mL	21.6 ± 9.1	-	<0.001
1,25(OH)$_2$D, pg/mL	45.2 ± 19.0	<0.001	-
Demographics			
Age, years	52 ± 12	0.14	0.19
Male gender, n (%)	222 (51.0)	0.02	0.01
Current smoker, n (%)	93 (21.4)	0.03	<0.001
Current diabetes, n (%)	76 (17.5)	0.002	0.59
BMI, kg/m^2	25.9 ± 4.3	0.001	0.13
Waist circumference, cm	97 ± 14	0.003	0.003
Systolic blood pressure, mmHg	153 ± 24	0.02	0.02
Diastolic blood pressure, mmHg	90 ± 10	0.16	0.03
Transplantation			
Transplant vintage, years	6.3 [3.1-11.7]	0.14	0.01
Deceased donor, n (%)	383 (88.0)	0.62	0.91
Dialysis duration, months	28 [13-49]	0.001	0.43
Renal function			
Proteinuria, g/24h	0.2 [0.0-0.5]	0.56	<0.001
eGFR, CKD-EPI, mL/min/1.73m^2	46.5 ± 16.4	0.20	<0.001
Laboratory measurements			
Total cholesterol, mg/dL	217 ± 39	0.17	0.28
HDL cholesterol, mg/dL	43 ± 13	0.24	<0.001
LDL cholesterol, mg/dL	136 ± 37	0.009	<0.05
C-reactive protein, mg/L	2.2 [0.9-5.1]	<0.001	0.007
Serum albumin, g/dL	4.0 ± 0.3	0.04	0.03
Calcium, mg/dL	9.5 ± 0.6	0.82	0.03
Phosphate, mg/dL	3.3 ± 0.7	0.85	<0.001
FGF-23, RU/mL	142 [95-236]	0.46	<0.001
PTH, pg/mL	86 [56-125]	0.02	0.45
Medication			
ACEi or ARB, n (%)	150 (34.5)	0.68	<0.001
No. of antihypertensives	2 [1-3]	0.34	0.19
Vitamin D suppletion, n (%)	43 (9.9)	0.16	<0.05
Statins, n (%)	220 (50.6)	0.12	<0.001

Data are presented as n (%), mean ± SD, or median [interquartile range] for nominal, normally distributed, and non-normally distributed data, respectively. The *P*-value represents the *P* for trend in univariable linear regression analysis.
Abbreviations: 1,25(OH)$_2$D, 1,25 dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D; ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; BMI, body mass index; eGFR, estimated glomerular filtration rate; FGF-23, fibroblast growth factor-23; HDL, high-density lipoprotein; LDL, low density lipoprotein; PTH, parathyroid hormone.

mortality (hazard ratio [HR], 0.64; 95% confidence interval [CI], 0.51-0.81; *P*<.001 and 0.69 [95% CI, 0.55-0.87], *P*=.002 per SD increase, respectively) (Table 3). The inverse association of 25(OH)D with all-cause mortality remained significant after adjustment for potential confounders including age, sex, current smoking, systolic BP, waist circumference, diabetes mellitus, eGFR (CKD-EPI) and determinants of 25(OH)D (*i.e.*, season, LDL cholesterol, ln PTH and dialysis vintage) (HR, 0.68; 95% CI, 0.52-0.89; *P*=.004), whereas the association of 1,25(OH)$_2$D with all-cause mortality lost significance after adjustment for renal function (HR, 0.85 [95% CI, 0.66-1.10], *P*=.2) (Table 3). The

Table 2. Multivariable associations of 25(OH) vitamin D and 1,25$(OH)_2$ vitamin D concentrations with clinical parameters in stable renal transplant recipients

Clinical parameter	25(OH)D		1,25$(OH)_2$D	
	Std β	*P*-value	Std β	*P*-value
25(OH)D (ng/mL)	-	-	0.35	<0.001
1,25$(OH)_2$D (pg/mL)	0.30	<0.001	-	-
Male gender	0.19	<0.001	-	-
Season	0.30	<0.001	-	-
Smoking	-	-	- 0.09	0.02
Waist circumference, cm	- 0.14	0.001	-	-
eGFR, ml/min/1.73m^2	-	-	0.36	<0.001
Use of ACEi/ARB	-	-	- 0.11	0.004
HDL cholesterol, mg/dL	-	-	0.14	<0.001
LDL cholesterol, mg/dL	0.13	0.002	-	-
Use of statins	-	-	0.29	<0.001
Phosphate, mg/dL	-	-	- 0.13	0.002
Ln PTH, pg/mL	- 0.11	0.03	-	-
Transplant vintage, years	-	-	- 0.19	<0.001
Dialysis duration, months	- 0.10	0.02	-	-

Data are presented as standardized β coefficient (std β) with corresponding *P*-value for the pooled data.
Abbreviations: 1,25$(OH)_2$D, 1,25 dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D; ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PTH, parathyroid hormone.

Table 3. Associations of 25(OH)vitamin D and 1,25$(OH)_2$ vitamin D levels with all-cause mortality in stable renal transplant recipients (n_{events}/n_{total} = 99/435)

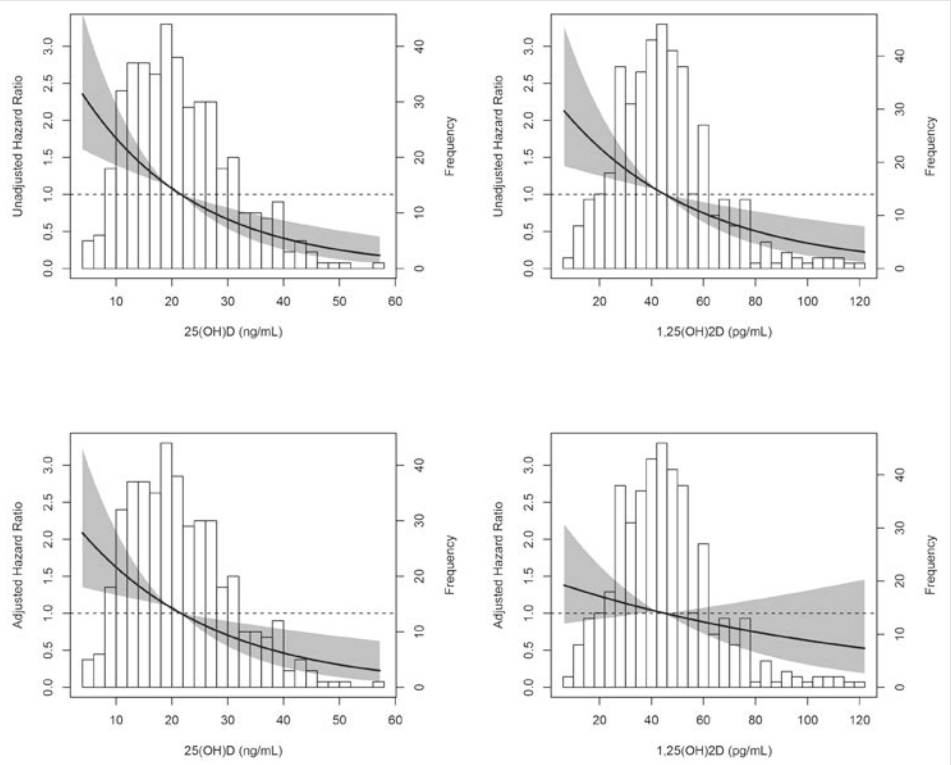
	25(OH)D			1,25$(OH)_2$D		
	HR	95% CI	*P*	HR	95% CI	*P*
Model 1	0.64	0.51 to 0.81	<0.001	0.69	0.55 to 0.87	0.002
Model 2	0.69	0.55 to 0.87	0.001	0.72	0.58 to 0.90	0.003
Model 3	0.73	0.57 to 0.93	0.01	-	-	-
Model 3a	-	-	-	0.77	0.61 to 0.97	0.03
Model 4	0.72	0.52 to 0.93	0.01	-	-	-
Model 4a	-	-	-	0.77	0.60 to 0.97	0.03
Model 5	0.68	0.52 to 0.89	0.004	-	-	-
Model 5a	-	-	-	0.85	0.66 to 1.10	0.2

Data are presented as hazard ratio (HR) per standard deviation increase in 25(OH)D or 1,25$(OH)_2$D concentrations plus 95% confidence interval (CI). Model 1: crude. Model 2: adjusted for age and gender. Model 3: as model 2 and additionally adjusted for current smoking, systolic blood pressure, waist circumference, LDL cholesterol, and diabetes mellitus. Model 3a: as model 2 and additionally adjusted for current smoking, systolic blood pressure, waist circumference, HDL cholesterol, and diabetes mellitus. Model 4: as model 3 and additionally adjusted for season, dialysis vintage, and ln PTH. Model 4a: as model 3a and additionally adjusted for use of ACEi/ARB, and transplant vintage. Model 5: as model 4 and additionally adjusted for eGFR. Model 5a: as model 4a and additionally adjusted for eGFR and serum phosphate.

crude and fully adjusted HRs for all-cause mortality according to levels of 25(OH)D and 1,25$(OH)_2$D are shown in Figure 1.

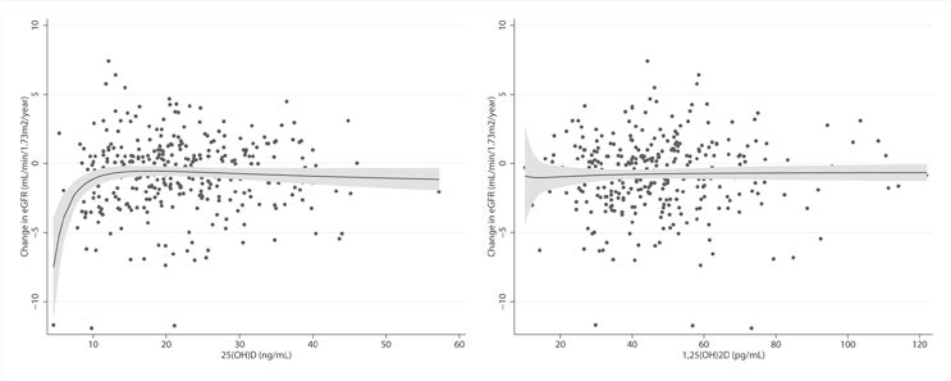
Cardiovascular disease was the most frequent cause of death (53%) followed by malignancy (25%) and infection (15%). In univariable analyses, the causes of death cardiovascular disease, and infection were inversely associated with 25(OH)D (HR, 0.67 [95% CI, 0.49-0.92]; *P*=.01; HR, 0.35 [95% CI, 0.17-0.73]; *P*=.005, respectively) whereas malignancy was not (Supplemental Table 2).

Figure 1. The risk for all-cause mortality presented by 25(OH)-vitamin D, left; and 1,25(OH)$_2$ vitamin D levels, right; compared with mean 25(OH)D and 1,25(OH)$_2$D concentrations, respectively, in stable renal transplant recipients



The line in the graph represents the risk for all-cause mortality. The grey area represents the 95% confidence interval of the hazard ratio (HR). The HRs of model 1 (crude) and the fully adjusted HRs (model 5 and 5a, respectively) are shown in the upper and lower figures, respectively.

Figure 2. Scatter plot of the annual change in eGFR according to 25(OH)D and 1,25(OH)$_2$D levels



The line in the graph indicates the predicted change in eGFR with corresponding 95% confidence interval. Data of one patient with extreme values of 1,25(OH)$_2$D (6.4 pg/mL) is not shown.

Table 4. Associations of 25(OH)vitamin D and 1,25$(OH)_2$ vitamin D levels with death-censored graft failure in stable renal transplant recipients (n_{events}/n_{total} = 44/435)

	25(OH)D			1,25$(OH)_2$D		
	HR	**95% CI**	***P***	**HR**	**95% CI**	***P***
Model 1	0.90	0.65 to 1.22	0.5	0.34	0.23 to 0.52	<0.001
Model 2	0.86	0.62 to 1.19	0.4	0.33	0.21 to 0.51	<0.001
Model 3	0.83	0.60 to 1.15	0.3	0.35	0.23 to 0.54	<0.001
Model 4	0.90	0.62 to 1.29	0.6	-	-	-
Model 4a	-	-	-	0.52	0.34 to 0.80	0.003
Model 5	0.92	0.79 to 1.09	0.6	0.77	0.49 to 1.21	0.3

Data are presented as hazard ratio (HR) per standard deviation increase in 25(OH)D and 1,25$(OH)_2$D concentrations plus 95% confidence interval (CI). Model 1: crude. Model 2: adjusted for age and gender. Model 3: adjusted for age, gender, HLA mismatches, age donor, gender donor, and type of transplantation. Model 4: adjusted for gender, season, waist circumference, LDL cholesterol, dialysis vintage and ln PTH. Model 4a: adjusted for current smoking, HDL cholesterol, use of ACEi/ARB, serum phosphate and transplant vintage. Model 5: adjusted for age, gender, eGFR and ln proteinuria.

As sensitivity analyses, we repeated Cox regression analyses in RTRs who did not use vitamin D supplements at baseline (n=392, 84 events). The results of these sensitivity analyses were not materially different from the primary analyses; 25(OH)D (fully adjusted HR, 0.68 [95% CI, 0.51-0.90] per SD increase; *P*=.008), but not 1,25$(OH)_2$D (fully adjusted HR, 0.86 [95% CI, 0.66-1.14] per SD increase; *P*=.3), was significantly associated with all-cause mortality after adjustment for potential confounders.

Vitamin D status, change in eGFR, and graft failure

In this population of stable RTRs, the mean annual change in eGFR was -0.76 ± 2.64 ml/min/1.73m^2/yr. Scatter plots of the annual change in eGFR and predicted annual change in eGFR according to levels of 25(OH)D and 1,25$(OH)_2$D are shown in Figure 2. In univariable linear regression analyses, 25(OH)D levels less than 12 ng/mL were significantly associated with a higher annual change in eGFR (β, -0.15; *P*=.007). This association remained significant after adjustment for potential confounders including age, sex, waist circumference, systolic BP, diabetes, eGFR, and ln proteinuria (β, -0.11; *P*=.03). After exclusion of subjects with vitamin D supplementation at baseline, we observed a similar trend in the fully adjusted analysis (β, -0.10; *P*= .06). In univariable linear regression analyses, 1,25$(OH)_2$D level was not associated with annual change in eGFR (β, 0.01; *P*=.8).

After a median follow-up of 7.0 (IQR, 5.8-7.5) years, 44 of 435 patients (10.1%) developed allograft failure. In univariable analyses, 1,25$(OH)_2$D, but not 25(OH)D, was significantly associated with death-censored graft failure (HR, 0.34 vs. 0.90; 95% CI, 0.23-0.52 vs. 0.65-1.22; *P*<.001 vs. =.5, respectively) (Table 4). However, the association of 1,25$(OH)_2$D with death-censored graft failure lost significance after adjustment for renal function (Table 4). The results of these sensitivity analyses in patients without vitamin D supplementation at baseline (n=392, 36 events) were not materially different from the primary analyses; 1,25$(OH)_2$D but not 25(OH)D was significantly associated with graft failure in univariable analyses (HR, 0.37 vs. 0.90; 95% CI, 0.24-0.59 vs. 0.63-1.27; *P*<.001 vs. =.5 per SD increase, respectively), but this association lost significance after adjustment for renal function (HR, 0.92; 95% CI, 0.57-1.48; *P*=.7 per SD increase).

DISCUSSION

The main aim of this study was to investigate the long-term implications of low 25(OH)D and 1,25$(OH)_2$D levels measured in stable RTRs greater than 1 year after transplantation. In a large observational cohort of 435 stable RTRs, we found that 25(OH)D levels were independently associated with all-cause mortality and that very low levels of 25(OH)D (*i.e.*, <12 ng/ml) were independently associated with a stronger annual decline in eGFR during long-term follow-up. Although the inverse association of 25(OH)D with all-cause mortality was independent of renal function (eGFR), the associations of 1,25$(OH)_2$D with all-cause mortality and graft failure were dependent on renal function.

A recently published study of *Bienaimé et al*(13) showed that low levels of 25(OH)D early post-transplantation (*i.e.*, 3 mo after transplantation) were associated with lower measured GFR 1 year post-transplantation. In extension to these findings, *Obi et al*[25] showed that severe vitamin D deficiency (*i.e.*, <12 ng/ml) predicts a rapid decline in eGFR in RTR at less than 10 years after transplantation. In agreement with the results of these previous studies, we found that 25(OH)D levels less than 12 ng/ml were independently and significantly associated with a higher annual renal function decline. We extend the findings of previous studies and showed that 25(OH)D was associated with all-cause mortality and renal function decline independently of the time since transplantation.

In this study population, we also observed inverse associations of 1,25$(OH)_2$D with all-cause mortality and graft failure; however, these associations were dependent on renal function. This is not unexpected given the fact that the enzyme 1α-hydroxylase, the enzyme that converts 25(OH)D into 1,25$(OH)_2$D, is predominately expressed in renal tubular epithelial cells. Interestingly, the inverse association between 25(OH)D and all-cause mortality was independent of renal function or 1,25$(OH)_2$D. Besides tubular epithelial cells, various extrarenal tissues express 1α-hydroxylase; it is therefore conceivable that part of the circulating 25(OH)D is locally converted to 1,25$(OH)_2$D, with subsequent local activation of vitamin D receptors.[26]

In the general population and in patients with chronic kidney disease (CKD), preliminary intervention studies suggest that restoring 25(OH)D levels with nutritional supplementation might reduce the risk of mortality.[27,28] In patients with CKD, vitamin D analogs reduce proteinuria, an intermediate for renal and cardiovascular outcomes.[29] Several studies have shown that cholecalciferol supplementation safely increases 25(OH)D levels in renal transplant recipients. However, the limited sample size of these studies precludes conclusions on whether vitamin D can affect clinical outcomes.[30,31] The findings of this study, along with previous studies, urge for a large-scale trial in the renal transplant population addressing the relationship between vitamin D levels and patient and graft survival.

Several limitations of our study warrant consideration. First, our study was observational in nature, and although we adjusted for several potential confounding variables including parameters of renal function, the possibility of residual confounding cannot be excluded. Second, 25(OH)D and 1,25$(OH)_2$D levels were measured at a single time point only and therefore we could

not take potential changes over time into account. However, when intra-individual variability of variables is taken into account (*i.e.*, by repeated measurements) it would only strengthen the association between the variable and outcomes.[32,33] Furthermore, the number of patients with severe 25(OH)D deficiency [*i.e.*, 25(OH)D levels <12 ng/ml] was relatively small in this study population. Finally, because we were interested in the relationship between vitamin D status and outcome in stable RTRs and included patients greater than 1 year after transplantation, we could have introduced survivor bias. Although this limits the generalizability of our study to the overall transplant population, our cohort reflects the stable transplant recipient population, which is the vast majority of renal transplant recipients.

Given the observational design of our study, we can only speculate on the possible mechanisms involved in the inverse association between 25(OH)D levels and mortality. Vitamin D has been implicated in innate immunity; therefore vitamin D deficiency may contribute to an increased risk of infectious diseases, which are in any case a serious threat for RTRs.[34,35] Furthermore, prospective cohort studies demonstrated associations between vitamin D deficiency and increased risk of cancer or incident cardiovascular disease, independently of established risk factors.[36,37] The currently ongoing Vitamin D and Omega-3 Trial study (ClinicalTrials.gov NCT01169259) aims to enroll 20 000 subjects to prospectively investigate the effect of cholecalciferol in the primary prevention of cancer and cardiovascular disease.

The main strength of this study is that it is, to our knowledge, the first study to address the association of both 25(OH)D and 1,25$(OH)_2$D, with graft and patients outcomes in stable RTRs. Additional strengths are the relatively large cohort size, the long term, and complete follow-up. Finally, the availability of both 25(OH)D and 1,25$(OH)_2$D levels allowed a thorough comparison between 25(OH)D and 1,25$(OH)_2$D with long-term outcomes.

In conclusion, this study shows that low plasma 25(OH)D is independently associated with an increased risk of all-cause mortality in stable renal transplant recipients, thereby extending findings from other studies linking vitamin D deficiency with increased mortality in the general population, patients with CKD, and hemodialysis patients.[38-40] In addition, we found that low levels of 25(OH)D (*i.e.*, <12 ng/ml) were independently associated with higher annual decline in eGFR. These results should encourage randomized controlled trials evaluating the effect of vitamin D supplementation on long-term survival outcomes after kidney transplantation.

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DISCLOSURES

The authors have nothing to disclose.

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Supplementary Table S1. Baseline patient characteristics of the study population presented as tertiles of 25(OH)D and $1,25(OH)_2D$

	Tertiles of 25(OH)D				Tertiles of $1,25(OH)_2D$			
	Tertile 1	**Tertile 2**	**Tertile 3**	***P*-value**	**Tertile 1**	**Tertile 2**	**Tertile 3**	***P*-value**
N	144	146	145	-	144	146	145	-
25(OH)D, ng/mL	<16.4	16.4-24.7	>24.7	-	18.8±8.1	21.9±8.9	24.1±9.6	<0.001
$1,25(OH)_2D$, pg/mL	38.8±16.4	45.2±16.8	51.4±21.4	<0.001	<36.2	36.2-50.0	>50.0	-
Demographics								
Age, years	54±12	51±12	52±12	0.122	52±12	53±12	50±12	0.297
Male gender, n (%)	61 (42)	76 (52)	85 (59)	0.021	82 (57)	76 (52)	64 (44)	0.089
Season	-	-	-	-	-	-	-	-
Winter, n (%)	45 (31)	38 (26)	13 (9)	<0.001	38 (26)	25 (17)	33 (23)	0.159
Spring, n (%)	51 (35)	33 (23)	15 (10)	<0.001	32 (22)	41 (28)	26 (18)	0.117
Summer, n (%)	9 (6)	28 (19)	52 (36)	<0.001	15 (10)	27 (18)	47 (32)	<0.001
Autumn, n (%)	39 (27)	47 (32)	65 (45)	0.005	59 (41)	53 (36)	39 (27)	0.038
Current smoker, n (%)	43 (30)	26 (18)	24 (17)	0.010	42 (29)	31 (21)	20 (14)	0.006
Current diabetes, n (%)	34 (24)	30 (21)	12 (8)	0.001	27 (19)	23 (16)	26 (18)	0.785
BMI, kg/m2	26.4±4.8	26.5±4.3	24.8±3.5	0.002	26.0±4.4	25.9±4.1	25.8±4.5	0.710
Waist circumference, cm	98.1±13.6	97.9±14.4	93.7±12.7	0.006	98.0±13.3	96.7±12.8	94.9±14.9	0.048
Systolic blood pressure, mmHg	156±24	152±23	150±23	0.019	155±24	153±25	149±21	0.017
Diastolic blood pressure, mmHg	90±10	90±10	88±10	0.124	91±10	89±10	89±10	0.102
History of MI, n (%)	11 (8)	10 (7)	16 (11)	0.398	10 (7)	14 (10)	13 (9)	0.732
History of TIA/CVA, n (%)	6 (4)	8 (5)	5 (3)	0.703	9 (6)	4 (3)	6 (4)	0.318
Transplantation								
Transplant vintage, years	6.0 [2.4-11.3]	6.0 [3.5-11.3]	7.4 [3.7-12.6]	0.197	7.0 [3.6-12.0]	6.0 [2.9-12.4]	6.0 [2.5-10.7]	0.122
Deceased donor, n (%)	129 (90)	127 (87)	127 (88)	0.776	129 (90)	126 (87)	128 (88)	0.686
Dialysis duration, months	31 [14-57]	30 [13-47]	23 [11-45]	0.034	26 [12-52]	28 [16-46]	29 [12-48]	0.955
Cold ischemia time, hours	22 [15-28]	21 [15-26]	22 [16-29]	0.465	21 [15-25]	23 [18-28]	22 [15-27]	0.202
Total warm ischemia time, minutes	35 [30-45]	36 [30-45]	35 [30-45]	0.924	35 [30-45]	36 [30-45]	36 [30-45]	0.887
Total no. of HLA mismatches	2 [1-3]	2 [0-2]	2 [1-2]	0.581	1 [0-2]	2 [1-3]	2 [1-3]	0.164
History of acute rejection, n (%)	59 (41)	64 (44)	61 (42)	0.883	63 (44)	65 (45)	56 (39)	0.542
Renal function								
Proteinuria, g/24h	0.2 [0.0-0.6]	0.2 [0.0-0.6]	0.2 [0.0-0.5]	0.614	0.3 [0.2-0.7]	0.2 [0.0-0.5]	0.2 [0.0-0.4]	<0.001
eGFR, CKD-EPI, mL/min/1.73m2	46.6±17.2	46.9±15.0	46.0±16.9	0.731	38.8±15.6	46.2±15.3	54.3±14.5	<0.001
Laboratory measurements								
Hemoglobin, g/dL	13.5±1.5	13.6±1.3	13.3±1.5	0.371	13.2±1.4	13.5±1.5	13.7±1.3	0.001
Glucose, mg/dL	89.6±25.9	87.7±20.0	82.4±13.2	0.003	87.0±21.7	84.8±15.6	87.9±23.5	0.728
Total cholesterol, mg/dL	217 [189-243]	208 [187-237]	217 [194-239]	0.351	215 [191-241]	217 [190-240]	212 [188-237]	0.663
HDL cholesterol, mg/dL	40.9 [32.8-49.3]	39.8 [35.4-50.3]	42.5 [32.8-53.1]	0.597	36.7 [29.7-46.6]	41.7 [35.4-49.5]	43.2 [36.1-54.4]	<0.001
LDL cholesterol, mg/dL	135 [115-154]	133 [113-157]	140 [118-162]	0.180	137 [119-159]	139 [116-162]	133 [111-149]	0.155
Triglycerides, mg/dL	178 [126-248]	169 [130-232]	154 [116-205]	0.064	173 [129-245]	162 [124-217]	160 [120-218]	0.245
C-reactive protein, mg/L	2.8 [1.4-6.2]	2.6 [0.9-7.3]	1.7 [0.6-3.6]	<0.001	3.4 [1.1-8.0]	1.9 [0.7-4.4]	1.9 [1.0-4.1]	0.003
Serum albumin, g/dL	4.0±0.4	4.1±0.3	4.1±0.3	0.033	4.0±0.3	4.1±0.4	4.1±0.3	0.014
Calcium, mg/dL	9.52±0.67	9.58±0.63	9.54±0.59	0.708	9.41±0.59	9.62±0.60	9.60±0.68	0.010
Phosphate, mg/dL	3.40±0.68	3.22±0.66	3.33±0.62	0.337	3.52±0.70	3.30±0.66	3.12±0.55	<0.001
FGF-23, RU/mL	140 [94-252]	143 [94-232]	143 [96-244]	0.959	181 [122-372]	139 [91-236]	113 [83-170]	<0.001
PTH, pg/mL	99 [62-152]	83 [55-126]	79 [50-111]	0.020	76 [48-121]	101 [67-145]	83 [56-114]	0.007
Medication								
ACEi or ARB, n (%)	45 (31)	61 (42)	44 (30)	0.074	59 (41)	57 (39)	34 (23)	0.003
Number of antihypertensives	2 [1-3]	2 [1-3]	2 [1-3]	0.222	2 [1-3]	2 [1-3]	2 [1-3]	0.568
Vitamin D suppletion, n (%)	18 (13)	13 (9)	12 (8)	0.431	18 (13)	16 (11)	9 (6)	0.174
Statins, n (%)	79 (55)	76 (52)	65 (45)	0.212	54 (38)	77 (53)	89 (61)	<0.001
Prednisone, dose (mg/day)	10 [7.5-10]	10 [7.5-10]	10 [7.5-10]	0.344	10 [7.5-10]	10 [8.8-10]	10 [7.5-10]	0.123
Calcineurin inhibitor, n (%)	118 (82)	115 (79)	104 (72)	0104	104 (72)	110 (76)	123 (85)	0.028
Proliferation inhibitor, n (%)	99 (69)	107 (73)	109 (75)	0.455	100 (69)	119 (82)	97 (67)	0.009
Sicrolimus, n (%)	3 (2)	1 (1)	2 (1)	0.594	0 (0)	2 (1)	4 (3)	0.133

Data are presented as n (%), mean ± SD, and median [interquartile range] for nominal, normally distributed, and non-normally distributed data, respectively. The P-value represents the P for trend in univariable linear regression analysis.

Abbreviations: $1,25(OH)_2D$, 1,25 dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D; ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; BMI, body mass index; CVA, cerebrovascular accident; eGFR, estimated glomerular filtration rate; FGF-23, fibroblast growth factor-23; HDL, high-density lipoprotein; HLA, human leukocyte antigen; LDL, low density lipoprotein; MI, myocardial infarction; PTH, parathyroid hormone; TIA, transient ischemic attack.

Supplementary Table S2. Univariate associations of 25(OH)D with cause of death in stable renal transplant recipients

Cause of death	N	HR	95% CI	*P*
All-cause	99	0.64	0.51 to 0.81	<0.001
Cardiovascular	52 (53%)	0.67	0.49 to 0.92	0.01
Malignancy	25 (25%)	0.83	0.55 to 1.27	0.4
Infection	15 (15%)	0.35	0.17 to 0.73	0.005
Other/Unknown	7 (7%)	0.42	0.15 to 1.15	0.09

Data are presented as hazard ratio (HR) per standard deviation (SD) increase in 25(OH)D concentrations plus 95% confidence interval (CI). Mean ± SD 25(OH)D level: 21.6 ± 9.1 ng/mL.